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Glial cell line-derived neurotrophic factor (GDNF) is an endogenous protector in the mesolimbic system against excessive alcohol consumption and relapse

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ABSTRACT

Moderate social consumption of alcohol is common; however, only a small percentage of individuals transit from social to excessive, uncontrolled alcohol drinking. This suggests the existence of protective mechanisms that prevent the development of alcohol addiction. Here, we tested the hypothesis that the glial cell line-derived neurotrophic factor (GDNF) in the mesolimbic system [e.g. the nucleus accumbens (Acb) and ventral tegmental area (VTA)] is part of such a mechanism. We found that *GDNF* knockdown, by infecting rat Acb neurons with a small hairpin RNA (shRNA) targeting the *GDNF* gene, produced a rapid escalation to excessive alcohol consumption and enhanced relapse to alcohol drinking. Conversely, viral-mediated overexpression of the growth factor in the mesolimbic system blocked the escalation from moderate to excessive alcohol drinking. To access the mechanism underlying GDNF's actions, we measured the firing rate of dopaminergic (DAergic) neurons in the VTA after a history of excessive alcohol intake with or without elevating GDNF levels. We found that the spontaneous firing rate of DAergic neurons in the VTA was reduced during alcohol withdrawal and that GDNF reversed this alcohol-induced DA deficiency. Together, our results suggest that endogenous GDNF in the mesolimbic system controls the transition from moderate to excessive alcohol drinking and relapse via reversal of alcohol-dependent neuro-adaptations in DAergic VTA neurons.

Keywords Alcohol addiction, dopamine, GDNF, nucleus accumbens, relapse, ventral tegmental area.

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INTRODUCTION

Alcohol abuse is a serious worldwide health problem with concomitant medical, social and economic burdens, for which pharmacotherapeutic approaches are very limited (World Health Organization 2004; RSA White Paper 2009; Spanagel 2009). Although many people consume moderate levels of alcohol throughout their lives, only a minority of individuals escalate to uncontrolled, long-term excessive consumption and, in some cases, dependence (World Health Organization 2004), suggesting the existence of mechanisms that protect against a transition from moderate to excessive alcohol drinking.

One potential factor that may play a role in such protective mechanisms is glial cell line-derived neurotrophic factor (GDNF), an essential growth factor for the development, survival of midbrain dopamine (DA) neurons (Lin *et al.* 1993; Airaksinen & Saarma 2002; Bespalov & Saarma 2007; Pascual *et al.* 2008). Recently, GDNF has been implicated as a negative regulator in addiction, including alcohol addiction (Carnicella & Ron 2009; Ghitza *et al.* 2010; Davies *et al.* 2013). Specifically, we previously found that activation of the GDNF pathway in the ventral tegmental area (VTA) reduces alcohol reward and consumption and abolishes relapse in rats (Carnicella *et al.* 2008; Carnicella, Amamoto & Ron 2009c; Barak

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et al. 2011a,b). We also found that mice with reduced endogenous GDNF levels (GDNF heterozygote knockout) exhibit higher levels of conditioned place preference to alcohol and increased alcohol intake following a period of abstinence compared with wild-type mice (Carnicella *et al*. 2009b), suggesting that endogenous GDNF regulates alcohol reward and relapse. More recently, we found that the levels of GDNF are altered in response to alcohol intake. Specifically, we found that *GDNF* levels were elevated in the VTA of rats that experienced 1 week of voluntary intake of 20 percent alcohol. Interestingly, we observed that knockdown of *GDNF* expression in the VTA at the early stages of alcohol drinking led to a significant increase in alcohol consumption (Ahmadiantehrani, Barak & Ron 2013). In contrast, we found that 24-hour withdrawal after intermittent access to 20 percent alcohol for 7 weeks led to reduction in *GDNF* expression in the VTA (Ahmadiantehrani *et al*. 2013). Finally, we showed that infusion of GDNF into the VTA suppressed alcohol seeking and reward by normalizing DA levels in the nucleus accumbens (Acb) (Barak *et al*. 2011b). Interestingly, human alcoholics undergoing protracted withdrawal show reduced levels of GDNF in the blood serum (Heberlein *et al*. 2010). Taken together, our findings suggest that GDNF in the mesolimbic system is an endogenous alcohol-responsive gene that acts to dampen the adverse phenotypes associated with alcohol exposure. Here, we set out to test this hypothesis by determining whether, and if so how, GDNF gates the transition from moderate to excessive alcohol consumption and relapse to alcohol seeking and drinking.

MATERIALS AND METHODS

See Supporting Information Appendix S1 for details about reagents, cloning and preparation of viruses, intracerebral infusion of GDNF and viruses, semi-quantitative RT-PCR, immunohistochemistry and electrophysiology.

Animals

Male Long-Evans rats (Harlan, Indianapolis, IN; 300–450 g) were housed under a 12-hour light/dark cycle (lights on at 7:00 a.m.) with food and water available *ad libitum*. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Intermittent access to 20 percent alcohol in the two-bottle choice drinking procedure

The intermittent access to alcohol was performed as previously described (Carnicella *et al*. 2008, 2009c). In virally mediated gene delivery experiments, the procedure began 7 days after infection with Adv-shGDNF or 28 days after the infection with AAV2-GDNF. Specifically, animals were given 24 hours of concurrent access to one bottle of 20 percent (vol/vol) alcohol in tap water and one bottle of water, starting at 11:00 a.m. on Monday, Wednesday and Friday, with 24 or 48 hours of alcohol deprivation periods in between the alcohol-drinking sessions. The placement (left or right) of each solution was alternated between each session to control for side preference. The water and alcohol bottles were weighed after 30 minutes, 4 hours and 24 hours of access, according to the experimental protocol. A bottle containing water in a cage without rats was used to evaluate the spillage due to the experimental manipulations during the test sessions. Spillage was always ≤0.5 ml (<2.5 percent of the total fluid intake). The procedure was terminated after 21 alcohol access sessions, when control rats maintained a stable baseline of alcohol consumption of 5.5–6 g/kg/24 hours.

Continuous access to 10 percent alcohol in the two-bottle choice drinking procedure

The continuous access to alcohol was performed as previously described (McGough *et al*. 2004). Specifically, animals were given 24 hours of concurrent access to one bottle of 10 percent (vol/vol) alcohol in tap water and one bottle of water, continuously. The placement (left or right) of each solution was alternated every 24 or 48 hours to control for side preference. The water and alcohol bottles were weighed daily to monitor fluid intake. A bottle containing water in a cage without rats was used to evaluate the spillage due to the experimental manipulations during the test sessions. Spillage was always ≤0.5 ml (<2.5 percent of the total fluid intake). In the abstinence period, the alcohol bottle was removed such that rats had access only to water.

Operant alcohol or sucrose self-administration

The procedure was conducted as previously described (Carnicella *et al*. 2008; Jeanblanc *et al*. 2009). In the alcohol self-administration experiments, rats were habituated to drinking alcohol in their home cages by exposure to 10 percent alcohol in tap water (vol/vol) in a continuous access to two-bottle choice procedure for 3 weeks. Operant alcohol self-administration training then commenced. The self-administration chambers contain two levers: an active (alcohol or sucrose) lever, where pressing the lever resulted in delivery of 0.1 ml of a 10 percent alcohol or 2 percent sucrose solution, and an inactive lever, for which responses are counted as a measure of non-specific behavioral activity but no programmed events occur. After two to three nights in the

chambers to allow acquisition of a lever-press response for the corresponding solution under a fixed ratio 1 (FR1; one press delivers one reward), 60-minute sessions were conducted 5 days per week, with the schedule requirement increased to FR3 over the first week.

Reinstatement and reacquisition of alcohol self-administration

Reinstatement refers to the relapse of the response after a single presentation of the reinforcer (Bouton 2002) and is considered a measure of relapse to seeking (Bouton 2002), whereas reacquisition is the rapid return of responding when the outcome is made available again after a period of extinction and is a measure of relapse to consumption (Ricker & Bouton 1996; Bouton 2002) that is especially relevant for therapies that seek to extinguish drug-related behaviors (Kehoe & Macrae 1997). After 6 weeks of alcohol self-administration training, rats underwent daily 60-minute extinction sessions (no reward after active lever responses). After 10 days of extinction, the Acb of the rats was infected with Adv-shGDNF or Adv-shSCR. Eight and nine days after the surgery, relapse to alcohol seeking and drinking was tested in reinstatement and reacquisition sessions, respectively. To obtain a one-session reinstatement and reacquisition, the retrieval of operant alcohol self-administration was triggered by a prime of a non-contingent delivery of alcohol (0.2 ml, 10 percent) in the reward port when the test session started, as previously described (Carnicella *et al.* 2008; Wang *et al.* 2010b).

Reinstatement

Rats were placed in the chambers for a 60-minute session, with no alcohol delivery after lever presses, similarly to the extinction training session. A 0.2-ml drop of 10 percent alcohol was delivered into the reward port non-contingently to the lever response when the session started (the alcohol prime).

Reacquisition

This session was identical to the reinstatement stage, except that a 0.1 ml alcohol reward was delivered following lever presses (at FR3) as in the training sessions (for experimental timeline, see Fig. 3a).

Statistical analysis

Data were analyzed by *t*-test or one- or two-way ANOVA with/without repeated measures, as specified. Alcohol/sucrose drinking experiments were conducted in a between-subject design. Student–Newman–Keuls *post hoc* analysis was used where indicated.

RESULTS

Downregulation of *GDNF* in the Acb facilitates the transition from moderate to excessive alcohol drinking

Repeated cycles of voluntary consumption of 20 percent alcohol and withdrawal in rats produces a gradual escalation from moderate alcohol intake (2–3 g/kg/24 hours) at the early stages of the process (first and second weeks) to excessive alcohol consumption (5–6.5 g/kg/24 hours) after 4–5 weeks of training (Wise 1973; Carnicella *et al.* 2008, 2009c; Simms *et al.* 2008). Thus, this procedure models the transition from moderate to excessive alcohol drinking (Simms *et al.* 2008; Carnicella *et al.* 2009c; Becker 2013; Carnicella, Ron & Barak 2014).

Therefore, we utilized this intermittent access procedure to test the hypothesis that GDNF is an endogenous protective agent that gates the transition from moderate to alcohol-drinking behaviors. A major source of GDNF in the mesolimbic system is the Acb; therefore, we first tested the role of the growth factor in this brain region on alcohol intake. In order to focally downregulate the expression of *GDNF*, Acb of rats were infected with adenovirus (Adv) expressing short hairpin (sh)GDNF (1.3×10^9 TU/ml) or with a control virus expressing a scrambled sequence (Adv-shSCR). *In vivo* infection of neurons with Adv-shGDNF produces a transient and robust (~60 percent) decrease in *GDNF* expression at the infected region, which is detected 7–25 days post-virus infusion (Wang *et al.* 2010a; Barak *et al.* 2011a). Therefore, rats began their training in the intermittent access to 20 percent alcohol procedure, 7 days after viral infection and continued to drink for 7 weeks thereafter.

Control rats infected with Adv-shSCR in the Acb showed moderate alcohol intake levels at the beginning of the procedure (~2–3.5 g/kg/24 hours), and gradually escalated to excessive alcohol drinking (>4.5 g/kg/24 hours) over 3–4 weeks of training (Fig. 1a). In contrast, rats with reduced levels of *GDNF* in the Acb consumed excessive alcohol amounts as early as the third drinking session (first week), and alcohol intake levels were significantly higher than control rats until the eighth session, i.e. 4 weeks post-virus infusion [Fig. 1a; two-way mixed model ANOVA, Viral infection × Sessions interaction [$F_{(17,255)} = 4.85$, $P < 0.0001$], *post hoc* analysis using method of contrasts revealed a difference between groups in days 9–27 post-viral infection ($P < 0.001$). Further analysis showed that Adv-shGDNF-infected rats consumed more alcohol than control rats in the first week, but not in the sixth week {Fig. 1b; Viral infection × Time interaction [$F_{(1,15)} = 13.95$, $P < 0.002$, *post hoc* comparisons: difference between Adv-shGDNF- and Adv-shSCR-infected groups in the first week ($P < 0.007$) but not in the sixth week ($P = 0.94$)}. Notably, this temporary effect correlates well with the transient nature of

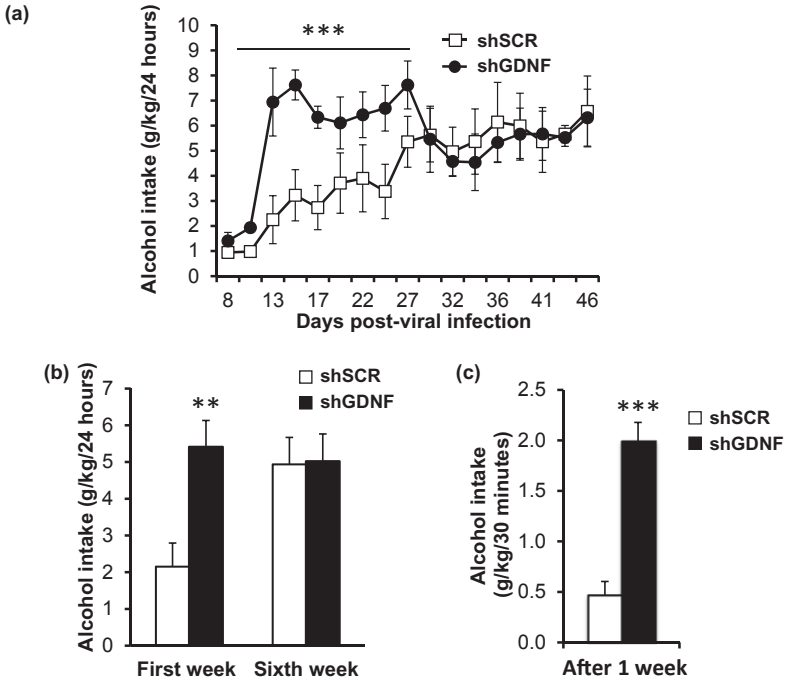

Figure 1 Downregulation of glial cell line-derived neurotrophic factor (*GDNF*) expression in the nucleus accumbens (Acb) facilitates the transition from moderate to excessive alcohol consumption. (a–c) Adv-shGDNF (shGDNF) or Adv-shSCR (shSCR; 1.3×10^9 TU/ml; total of 3 µl/side) was infused into the Acb of rats to locally downregulate *GDNF* expression (Wang *et al.* 2010a). Eleven days later, rats began training in an intermittent access to 20 percent alcohol two-bottle choice procedure. (a) Alcohol intake throughout the 6 weeks of training. (b) Average alcohol intake during the first or the sixth week of training of rats infected with Adv-shGDNF or Adv-shSCR. (c) Alcohol intake during the initial 30 minutes of the fourth drinking session (i.e. after 1 week of training). Data are expressed as mean ± SEM of alcohol intake during 24 hours (a, b) or 30 minutes (c), in g/kg. $n = 8$–9. $^{**}P < 0.01$; $^{***}P < 0.001$

adenoviral-mediated gene delivery and expression (Ghosh, Gopinath & Ramesh 2006) and with the time course of Adv-shGDNF knockdown of GDNF (Wang *et al.* 2010a; Barak *et al.* 2011a). These results suggest that endogenous GDNF in the Acb protects against early transition to excessive alcohol drinking.

Upon reaching high intake levels (after 4 weeks of training), rats consume approximately 25 percent of the alcohol during the first 30 minutes of the 24-hour session (Simms *et al.* 2008; Carnicella *et al.* 2009c). This high level of alcohol consumption over a short time (1.5–2 g/kg/30 minutes) generates blood alcohol concentration (BAC) of >80 mg percent (Carnicella *et al.* 2009c), which meets the criteria of the National Institute on Alcohol Abuse and Alcoholism as binge drinking in humans (National Institute on Alcohol Abuse and Alcoholism 2004). As shown in Fig. 1c, after only 1 week of training, animals with decreased levels of *GDNF* in the Acb exhibited binge-like drinking during the first 30 minutes of the session, consuming levels usually seen only after 4–5 weeks of training (Carnicella *et al.* 2009c), whereas control rats infected with Adv-shSCR showed significantly lower levels of alcohol intake [$t(15) = 5.49$, $P < 0.0001$]. These results indicate that endogenous GDNF in the Acb plays a protective role against the development of binge-like drinking behavior.

To confirm that the endogenous GDNF system in the Acb serves to control the level of alcohol drinking, we trained a different group of rats to lever press for a 10 percent alcohol solution; rats consume moderate alcohol levels in this operant self-administration procedure, as previously described (Carnicella *et al.* 2008; Jeanblanc *et al.* 2009). After achieving a stable baseline response (average: 70–80 active lever presses), the Acb of rats was infected with Adv-shGDNF or control Adv-shSCR, as described earlier. We found that rats with reduced endogenous levels of *GDNF* in the Acb showed a 50 percent increase in the number of lever presses for the 10 percent alcohol reward {Fig. 2a; two-way mixed model ANOVA, Viral infection × Sessions interaction [$F_{(13,143)} = 2.25$, $P < 0.011$]; method of contrasts: difference between Adv-shGDNF and Adv-shSCR groups after ($P < 0.05$), but not before ($P = 0.73$) viral infection}, as well as in alcohol intake levels {Fig. 2b, Viral infection × Sessions interaction [$F_{(13,143)} = 2.49$, $P < 0.005$]; method of contrasts: difference between Adv-shGDNF and Adv-shSCR groups after ($P < 0.04$), but not before ($P = 0.67$) viral infection}. Importantly, no differences in the inactive lever presses were observed between the two groups (Supporting Information Fig. S1; $P > 0.05$), indicating that the increase in lever pressing was specific to the alcohol-associated lever. These findings also suggest that the memory for the lever-reinforcer association was intact and that there was no general effect on locomotor activity, in line with our previous report (Barak *et al.* 2011c). Taken together, these results further indicate that endogenous *GDNF* in the Acb protects against the development of excessive alcohol consumption.

Although our results suggest that endogenous *GDNF* in the Acb negatively regulates alcohol consumption, it is also possible that GDNF regulates general motivation to respond for rewards. To rule out this possibility, a similar

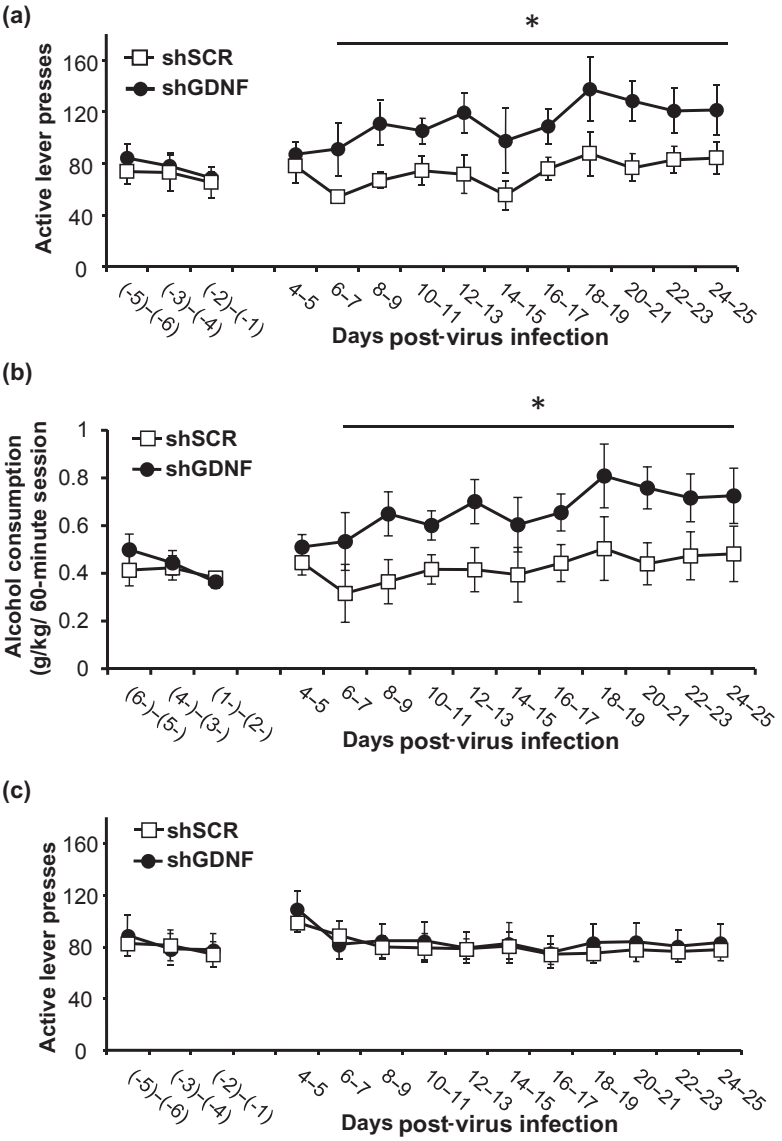

Figure 2 Downregulation of glial cell line-derived neurotrophic factor (*GDNF*) expression in the nucleus accumbens (Acb) results in an increased operant self-administration of alcohol, but not sucrose. (a–c) Rats were trained to press a lever for an alcohol (10 percent, a–b) or sucrose (2 percent, c) solution during a daily 60-minute session. After achieving a stable baseline operant responding, Adv-shGDNF (shGDNF) or Adv-shSCR (shSCR; 1.3×10^9 TU/ml; total of 3 µl/side) was infused into the Acb of rats to locally downregulate *GDNF* expression (Wang *et al.* 2010a). Number of operant responses for alcohol reward (a) and the resulting alcohol intake (b) during the days before and after viral infection. Number of operant responses for sucrose reward (c). Data are expressed as mean ± SEM. $n = 7–8$. *$P < 0.05$

experiment was conducted in rats trained to self-administer a solution of 2 percent sucrose. We found that downregulation of *GDNF* had no effects on the self-administration of this natural reward {Fig. 2c, no Sessions × Viral infection interaction [$F_{(13,156)} = 0.74$, $P = 0.73$]}. Taken together, these results suggest that endogenous *GDNF* in the Acb suppresses the transition from moderate to excessive alcohol consumption but does not play a role in the general motivation to consume rewards.

Downregulation of *GDNF* in the Acb increases relapse to alcohol seeking and drinking

Next, we tested whether a focal downregulation of *GDNF* within the Acb affects the reinstatement and reacquisition of operant responding for alcohol after extinction (timeline; Fig. 3a), two behaviors considered to model relapse to alcohol seeking (Le & Shaham 2002; Wang *et al.* 2010b; Barak *et al.* 2013; Marchant, Li & Shaham 2013) and drinking (Kehoe & Macrae 1997; Carnicella *et al.* 2008; Barak *et al.* 2013; Marchant *et al.* 2013), respectively. We found that knockdown of *GDNF* in the Acb after extinction training increased operant responding in both the reinstatement and the reacquisition tests {Fig. 3b; two-way mixed model ANOVA, Viral infection × Stage [$F_{(3,39)} = 7.15$, $P < 0.001$]; *post hoc* comparisons: difference between Adv-shGDNF and Adv-shSCR groups in the reinstatement and reacquisition stages (P's < 0.01), but not in the baseline or extinction stages (P's > 0.79)}. Importantly, there was no effect on the inactive lever presses (Supporting Information Fig. S2). These results imply that endogenous GDNF restrains relapse to alcohol seeking and drinking, after a period of withdrawal.

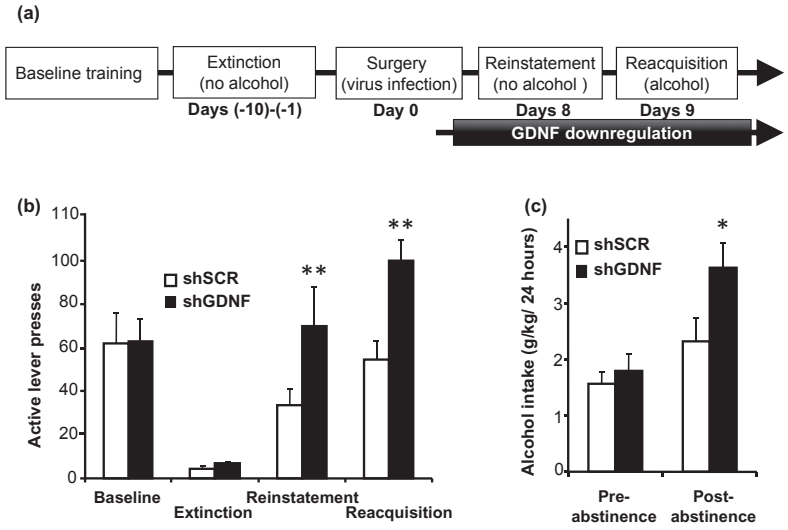

Figure 3 Downregulation of glial cell line-derived neurotrophic factor (*GDNF*) expression in the nucleus accumbens (Acb) enhances relapse to alcohol seeking and drinking. (a) A schematic timeline of the experiment. After achieving a stable baseline of alcohol (10 percent) self-administration rats underwent 10 days of extinction, after which Adv-shGDNF (shGDNF) or Adv-shSCR (shSCR; 1.3×10^9 TU/ml; total of 3 μl/side) was infused into the Acb of rats to locally downregulate *GDNF* expression (Wang *et al.* 2010a). On days 8 and 9, relapse was assessed: on day 8 post-viral infection, rats had a reinstatement test (alcohol was not delivered following lever presses as a measure of relapse to alcohol seeking). The next day, rats had a reacquisition test (alcohol was delivered following lever presses as a measure of relapse to alcohol consumption). A non-contingent alcohol prime was delivered at the beginning of these sessions. (b) Mean ± SEM of active lever presses. $n = 7$–8. (c) Rats were trained to consume alcohol (10 percent) in a continuous access to two-bottle choice procedure. After achieving a stable baseline of alcohol (10 percent) consumption, the Acb was infected by Adv-shGDNF or Adv-shSCR. After 7 days of abstinence, alcohol relapse was assessed by a 24-hour two-bottle choice drinking session. Data are expressed as mean ± SEM of alcohol intake during 24 hours, in g/kg. $n = 7$–9. $^*P < 0.05$; $^{**}P < 0.01$

To further investigate this possibility, we tested the outcome of *GDNF* knockdown in the Acb in another relapse model, taking advantage of the alcohol deprivation effect (Sinclair & Senter 1968; Spanagel & Holter 1999; Le & Shaham 2002). Rats were trained to consume moderate levels of alcohol in their home cage with concomitant continuous access to two bottles (10 percent alcohol and water) and were then subjected to a week of alcohol abstinence. Relapse to alcohol was then tested in a 24-hour drinking session. We found that Adv-shGDNF-infected rats showed higher levels of alcohol intake after abstinence compared to their control counterparts {Fig. 3c; mixed model two-way ANOVA, Viral infection × Abstinence interaction [$F_{(1,14)} = 5.74$, $P < 0.05$], *post hoc* comparisons: difference between Adv-shGDNF- and Adv-shSCR groups after ($P < 0.025$) but not before ($P = 0.51$) abstinence}. This finding confirms that endogenous *GDNF* in the Acb mitigates the intensity of relapse.

Upregulation of GDNF in the mesolimbic system prevents the transition from moderate to excessive alcohol drinking

If downregulation of endogenous GDNF facilitates the transition from moderate to excessive alcohol consumption, then an increase in GDNF levels should impede this transition. Therefore, in the next experiment, we tested whether long-term overexpression of GDNF in the Acb would prevent or hinder transition from moderate to excessive alcohol consumption.

The Acb of rats were infected with AAV2-GDNF (Johnston *et al.* 2009; Ciesielska *et al.* 2011), which resulted in a significant increase in the levels of the growth factor as compared to rats infected with control AAV2-GFP [Supporting Information Fig. S3; *t*-test showing increased *GDNF* expression in the Acb of AAV-GDNF-infected rats $t(7) = 3.96$, $P < 0.01$]. Rats were then trained in the intermittent access to 20 percent alcohol two-bottle choice procedure, as described earlier. As expected, we found that control rats showed moderate levels of alcohol intake at the early stages of the procedure, but escalated to excessive alcohol intake with the progression of training (Fig. 4a). In contrast, rats overexpressing *GDNF* in the Acb showed much lower levels of alcohol intake throughout most of the experiment, and never escalated to excessive alcohol consumption {Fig. 4a; two-way mixed model ANOVA, main effects of Viral infection [$F_{(1,14)} = 14.89$, $P < 0.002$] and Sessions [$F_{(17,238)} = 8.06$, $P < 0.001$] as well as a significant interaction [$F_{(17,238)} = 1.91$, $P < 0.02$]; *post hoc* analysis using method of contrasts revealed a difference between

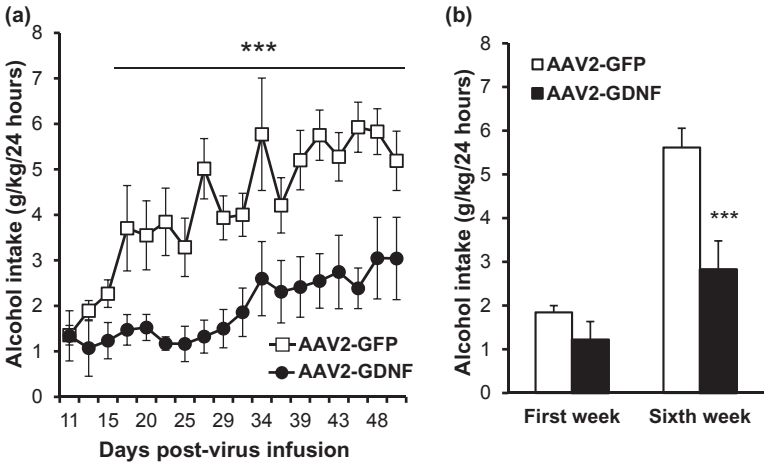

Figure 4 Upregulation of glial cell line-derived neurotrophic factor (*GDNF*) expression in the nucleus accumbens (Acb) blocks the transition from moderate to excessive alcohol consumption. (a, b) AAV2-GDNF or control AAV2-GFP (1×10^{12} TU/ml; total 3 μl/side) was infused into the Acb of rats to upregulate the expression of *GDNF*. Eleven days later, rats began training in an intermittent access to 20 percent alcohol two-bottle choice procedure. (a) Alcohol intake throughout the 6 weeks of training. (b) Average alcohol intake during the first or sixth week of training of rats infected with AAV2-GDNF or AAV2-GFP. Data are expressed as mean ± SEM of alcohol intake during 24 hours, in g/kg. $n = 8$–9. ***$P < 0.001$

groups in days 15–50 post-viral infection ($P < 0.001$)}. Further analysis indicated that AAV2-GDNF-infected rats consumed similar amounts of alcohol as control rats during the first week, but in the sixth week, control rats consumed significantly higher amounts of alcohol {Fig. 4b; Viral infection × Time interaction [$F_{(1,15)} = 4.61$, $P < 0.05$], *post hoc* comparisons: difference between Adv-shGDNF- and Adv-shSCR-infected groups in the sixth week ($P < 0.001$), but not in the first week ($P = 0.35$) of training}. These results indicate that a long-term increase in *GDNF* levels in the Acb prevents the transition from moderate to excessive alcohol drinking.

GDNF is retrogradely transported by DA neurons from the Acb to the VTA where it binds to the receptor tyrosine kinase, Ret and the co-receptor GFRα1 receptor (Wang *et al*. 2010a). Thus, the effects of *GDNF* overexpression in the Acb on alcohol drinking escalation should be mediated by actions of the growth factor in the VTA. Therefore, we next tested whether overexpression of *GDNF* in the VTA yields results similar to those observed with overexpression of the growth factor in the Acb. We found that AAV2-GDNF-mediated overexpression of *GDNF* in the VTA [Supporting Information Fig. S4; *t*-test showing increased *GDNF* expression $t(10) = 4.01$, $P < 0.005$] prevented the transition to excessive alcohol consumption {Fig. 5a; two-way mixed model ANOVA, Viral infection × Session interaction [$F_{(17,221)} = 1.94$, $P < 0.02$] *post hoc* analysis using method of contrasts revealed a difference between groups in days 25–50 post-viral infection ($P < 0.01$)}. Specifically, AAV2-GDNF-infected rats consumed similar amounts of alcohol as control rats during the first week, but in the sixth week, control rats consumed significantly higher alcohol levels {Fig. 5b; Viral infection × Time interaction [$F_{(1,14)} = 10.35$, $P < 0.01$], *post hoc* comparisons: difference between Adv-shGDNF- and Adv-shSCR-infected groups in the sixth week ($P < 0.001$), but not in the first week ($P = 0.52$) of training}. Moreover, the levels of GDNF mRNA expression negatively correlated with the alcohol intake levels (Supporting Information Fig. S5). Taken together, our results suggest that overexpression of *GDNF* in the mesolimbic system prevents the transition from moderate to excessive alcohol consumption via actions of the growth factor in the VTA.

GDNF reverses neuro-adaptations in VTA dopaminergic neurons induced by prolonged alcohol exposure

Finally, we addressed the possible mechanism by which GDNF suppresses alcohol drinking in the VTA. GDNF is produced in striatal regions (Pochon *et al*. 1997; Barroso-Chinea *et al*. 2005) and is retrogradely transported from the striatum to the midbrain (e.g. Acb to VTA) (Wang *et al*. 2010a), where it upregulates DA transmission in mesocorticolimbic projections by enhancing the activity of DAergic neurons in the VTA (Wang *et al*. 2010a). Since overexpression of *GDNF* in the Acb prevents the development of excessive alcohol consumption (Fig. 4), we tested whether overexpression of the growth factor in the Acb would also enhance the firing rate of VTA DAergic neurons. As shown in Fig. 6a and b, the firing rate of VTA DAergic neurons was significantly higher in rats infected with AAV2-GDNF in the Acb compared to AAV2-GFP-infected controls [$t(51) = 5.08$, $P < 0.001$]. Thus, our findings suggest that GDNF may

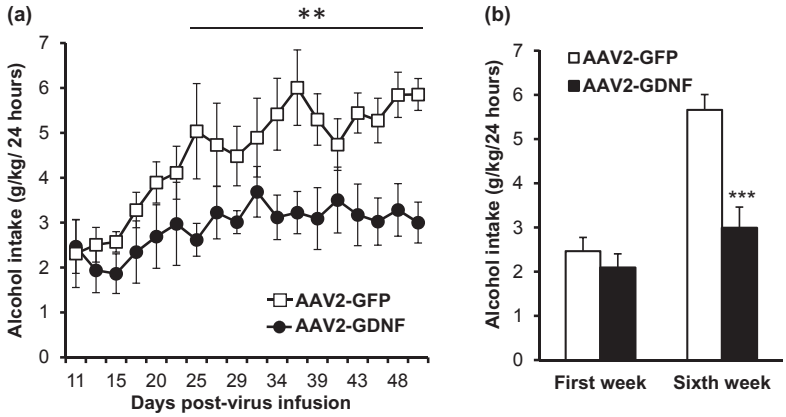

Figure 5 Upregulation of glial cell line-derived neurotrophic factor (*GDNF*) expression in the VTA blocks the transition from moderate to excessive alcohol consumption. (a, b) AAV2-GDNF or control AAV2-GFP (1×10^{12} TU/ml; 1.2 µl/side) was infused into the VTA of rats to upregulate the expression of *GDNF*. Eleven days later, rats began training in an intermittent access to 20 percent alcohol two-bottle choice procedure. (a) Alcohol intake throughout the 6 weeks of training. (b) Average alcohol intake during the first or the sixth week of training of rats infected with AAV2-GDNF or AAV2-GFP. Data are expressed as mean ± SEM of alcohol intake during 24 hours, in g/kg. $n = 8$. $^{**}P < 0.01$; $^{***}P < 0.001$

prevent the transition from moderate to excessive alcohol consumption by adjusting DA transmission in the mesolimbic system.

We previously found that long-term excessive alcohol consumption leads to downregulation of *GDNF* in the VTA (Ahmadiantehrani *et al.* 2013) and that viral-mediated knockdown of *GDNF* in the Acb leads to reduced spontaneous firing of DAergic neurons in the VTA (Wang *et al.* 2010a). Our present findings, showing that downregulation of *GDNF* in the Acb increases alcohol consumption and relapse, raise the possibility that these effects of GDNF are mediated by DAergic mechanisms. To confirm this suggestion, we next tested whether a long history of excessive alcohol consumption results in reduced spontaneous activity of VTA DAergic neurons (Wang *et al.* 2010a), and if so, whether this DAergic deficiency can be reversed by increasing GDNF levels. Rats were trained to consume high levels of alcohol in the intermittent access to 20 percent alcohol two-bottle choice procedure for 7 weeks as described earlier (average alcohol consumption: 5.50 ± 0.84 g/kg/24 hours). Midbrain slices were prepared after 24 hours of withdrawal and the firing rate of VTA DAergic neurons was recorded as previously described (Wang *et al.* 2010a). Alcohol-naïve, water-drinking animals served as the control group. We found that in rats with a history of excessive alcohol consumption, a 24-hour withdrawal caused a significant reduction in the spontaneous firing rate of VTA DA neurons, compared to water-drinking controls [Fig. 6c; $t(33) = 4.08$; $P < 0.001$]. Together with our previous reports (Barak *et al.* 2011b; Ahmadiantehrani *et al.* 2013), these results suggest that withdrawal from long-term excessive alcohol consumption causes physiological neuro-adaptations in the VTA, reflected by downregulation of *GDNF* expression and by reduced spontaneous firing in DAergic neurons, a reduction that results in deficient DA efflux in the Acb, and increased alcohol seeking and relapse (Barak *et al.* 2011b).

Since we previously showed that *in vivo* or *ex vivo* application of GDNF increases the spontaneous firing of Acb-projecting DAergic neurons in midbrain slices (Wang *et al.* 2010a), we next tested whether GDNF could reverse the deficient activity of VTA DA neurons induced by alcohol withdrawal. Rats were trained to consume excessive amounts of alcohol as described earlier (average alcohol consumption: 5.07 ± 0.54 g/kg/24 hours; alcohol-naïve rats served as controls). Twenty-four hours after the last drinking session, recombinant GDNF (rGDNF, 10 µg/side) or vehicle was infused into the VTA, 10 minutes after infusion, midbrain slices were prepared and the spontaneous activity of DA VTA neurons was recorded as previously described (Wang *et al.* 2010a). Similar to the results shown in Fig. 6c, withdrawal from excessive alcohol consumption resulted in a reduced firing rate of DA VTA neurons (Fig. 6d). In contrast, intra-VTA infusion of rGDNF increased the firing activity of the neurons and completely abolished the deficiency in firing induced by repeated alcohol intake and withdrawal {Fig. 6d; two-way mixed model ANOVA, GDNF treatment × Alcohol exposure [$F_{(1,129)} = 3.98$, $P < 0.05$], *post hoc* comparisons: difference between the water and alcohol groups infused with vehicle ($P < 0.01$) but not when infused with GDNF ($P = 0.86$)}. Notably, GDNF also increased the firing rate in water-drinking control animals ($P < 0.005$), suggesting that GDNF is capable of affecting the normal, as well as the impaired, DAergic system. Together, these data suggest that alcohol-drinking induces deficiency in the DAergic mesolimbic

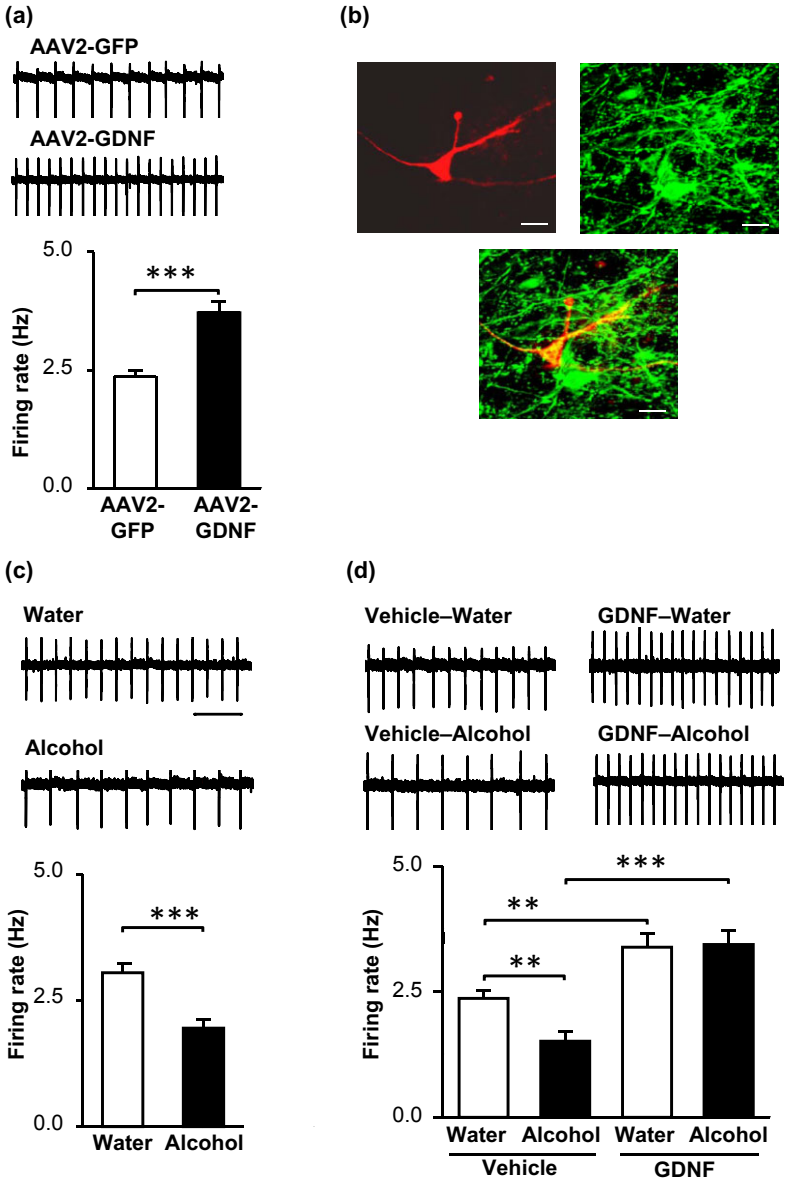

Figure 6 Glial cell line-derived neurotrophic factor (GDNF) reverses the inhibition of firing of ventral tegmental area (VTA) DAergic neurons induced by excessive alcohol consumption. (a) AAV2-GDNF or control AAV2-GFP (1×10^{12} TU/ml; total 3 µl/side) was infused into the nucleus accumbens (Acb) of rats to upregulate the expression of *GDNF*. Three weeks later, the spontaneous firing of VTA neurons was recorded in VTA slices. ***$P < 0.001$, $n = 28$ (AAV2-GFP) and 25 (AAV2-GDNF). Data are presented as mean ± SEM of spontaneous firing rate. (b) *Post hoc* TH-staining showing that recorded neurons were TH-positive. Whole-cell configuration was established after completing cell-attached recording and biocytin was infused through patch pipettes. Slices were then fixed to stain for TH and biocytin. Note that the biocytin-filled cell (red) is TH-positive (yellow). Scale bars, 20 µm (c) and (d). Rats were trained for 7 weeks to achieve a stable level of alcohol intake with the intermittent access to 20 percent alcohol in two-bottle choice procedure. Control animals consumed only water. (c) Following a 24-hour withdrawal period, VTA slices were prepared and the spontaneous firing of VTA neurons was recorded. ***$P < 0.001$, $n = 19$ (water) and 16 (alcohol). (d) Following a 24-hour withdrawal period GDNF (10 µg/side) or vehicle was infused into the VTA. Ten minutes later, brain slices were prepared and the spontaneous firing of VTA DAergic neurons was recorded. **$P < 0.01$; ***$P < 0.001$; $n = 32$ (water/vehicle), $n = 36$ (alcohol/vehicle), $n = 33$ (water/GDNF) and $n = 32$ (alcohol/GDNF)

system, and that GDNF antagonizes this deficiency by increasing the firing rate of VTA DAergic neurons.

DISCUSSION

Here, we describe a new role for the Acb-derived growth factor, GDNF, as an endogenous factor that protects against the transition from moderate to excessive alcohol consumption and gates relapse. Specifically, we show that small hairpin RNA (shRNA)-mediated knockdown of GDNF's expression in the Acb results in a very rapid escalation of alcohol intake and in a very sharp relapse after abstinence, whereas overexpression of *GDNF* in the mesolimbic system eliminates the escalation to excessive alcohol consumption. Finally, we demonstrate that GDNF rapidly reverses neuro-physiological adaptations in VTA DAergic neuron firing, induced by excessive alcohol consumption. Together, our results provide evidence that GDNF in the mesolimbic system is an endogenous factor that controls the transition from moderate to excessive alcohol consumption and relapse to alcohol seeking/drinking by modulating neuro-adaptations in the mesolimbic system (model, Fig. 7).

Downregulation of *GDNF* in the Acb led rats to consume very high levels of alcohol at the early stages of the intermittent access 20 percent alcohol two-bottle choice procedure. Strikingly, after only three sessions of training, Adv-shGDNF-treated rats showed binge-like alcohol drinking (2 g/kg/30 minutes). Such excessive alcohol consumption is typically seen in this procedure only after approximately 15 sessions of training, generating high BAC of about 115 mg percent (Carnicella *et al.*

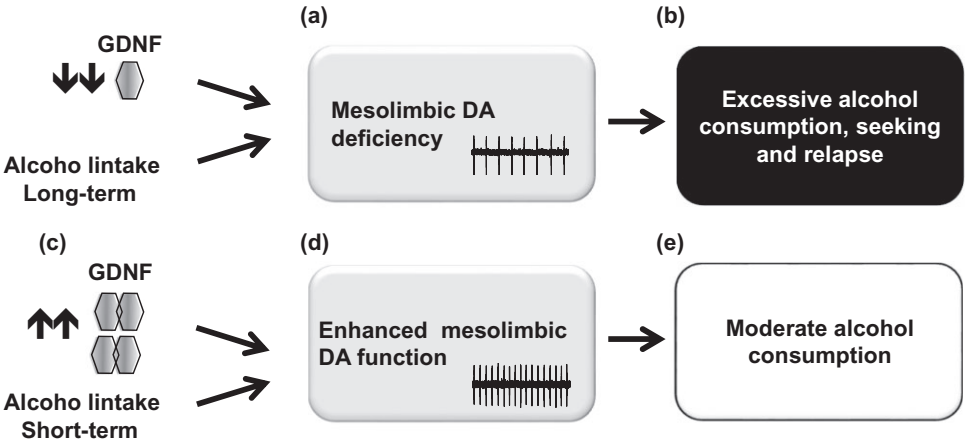

Figure 7 A model illustrating the effects of excessive alcohol consumption and of glial cell line-derived neurotrophic factor (GDNF) on alcohol drinking via actions on the dopamine (DA) mesolimbic system. Withdrawal from excessive alcohol consumption and downregulation of GDNF in the nucleus accumbens (Acb) result in deficient DAergic function in the mesolimbic system (a). This deficiency enhances alcohol seeking and intake (b), and alcohol temporarily alleviates the DA deficiency (Barak *et al.* 2011b). Increasing GDNF levels (c), either by infusion of rGDNF or by overexpressing GDNF in the mesolimbic system, reverses withdrawal-induced DA deficiency (d) and consequently suppresses alcohol seeking and drinking (e)

2009c). For comparison, control rats consumed considerably lower alcohol levels at this timepoint (0.5 g/kg/30 minutes), which leads to a much lower BAC (~25 mg percent) (Carnicella *et al.* 2009c). These results show that in the absence of normal *GDNF* expression in the mesolimbic system, rat's transition to excessive alcohol drinking occurs considerably earlier than controls.

Interestingly, we found that downregulation of *GDNF* in the Acb leads to a significant increase in alcohol consumption in rats trained to self-administer moderate levels of alcohol in an operant procedure. This procedure is considered to model moderate 'social' drinking (Carnicella *et al.* 2008; Jeanblanc *et al.* 2009), as the levels of alcohol consumption (~0.4 g/kg over 60-minute session) generate low levels of BAC of ~10 mg percent (Jeanblanc *et al.* 2009). Thus, these findings strengthen the possibility that endogenous GDNF protects social drinkers from escalating to excessive levels of alcohol consumption. Importantly, we show that sucrose consumption is not affected by downregulation of *GDNF* in the Acb. This finding, taken together with our previous finding that intra-VTA GDNF infusion has no effects on operant sucrose self-administration (Carnicella *et al.* 2008), indicates that the consequences of *GDNF* knockdown on alcohol self-administration were not due to a general increase in the motivation to press the lever for a reward, nor was the effect due to enhanced locomotor activity, suggesting that GDNF selectively regulates alcohol intake but not the intake of natural rewards. Furthermore, knockdown of the endogenous *GDNF* in the Acb remarkably increased relapse to alcohol seeking and drinking. These results are a reminiscent of the finding that GDNF heterozygote knockout mice show higher levels of alcohol intake after a period of abstinence compared to wild-type mice (Carnicella *et al.* 2009b). However, because *GDNF* was focally downregulated in the Acb, the present findings identified the mesolimbic system as the pathway in which endogenous GDNF acts to suppress alcohol-drinking behaviors. We previously demonstrated that a single infusion of rGDNF into the VTA suppresses alcohol consumption in models of moderate or excessive alcohol drinking (Carnicella *et al.* 2008, 2009c). Here, we show that prolonged overexpression of the growth factor in the Acb or VTA leads to moderate levels of alcohol consumption throughout the whole training session and prevents the escalation to excessive drinking. These findings suggest that while the protective effect of endogenous GDNF loses efficacy after recurring cycles of alcohol consumption and withdrawal, augmentation of this protection by viral-mediated overexpression of *GDNF* can block the escalation to excessive drinking.

GDNF is a protective neurotrophic factor that has the ability to support the survival of dopaminergic and motor neurons (Lindholm *et al.* 2007). With the levels of alcohol consumed by rats in the present study, we did not observe neuronal damage in rats with GDNF knockdown. Furthermore, we previously showed that knockdown of GDNF does not produce neuronal cell death (Wang *et al.* 2010a). However, GDNF also regulates the activity of DAergic neurons in the VTA as well as DA release in the Acb (Wang *et al.* 2010a). We report here that long-term upregulation of *GDNF* in the Acb increases the spontaneous firing of VTA DAergic neurons. We previously found that intra-VTA infusion of GDNF results in a similar effect and increases DA release in the Acb (Wang

et al. 2010a). Moreover, these increases suppress alcohol seeking and drinking by reversing alcohol withdrawal-induced DA deficiency in the Acb (Barak *et al*. 2011b). Thus, it is likely that *GDNF* overexpression in the Acb induces long-term increases in DA transmission in the mesolimbic system, which prevents the development of excessive alcohol consumption. Conversely, we show that withdrawal from excessive alcohol consumption leads to the opposite outcome—reduced firing of VTA DAergic neurons. We further demonstrate that withdrawal-induced decrease in VTA firing is rapidly reversed by a local infusion of rGDNF, which has also been shown to reduce alcohol drinking, seeking and relapse (Carnicella *et al*. 2008, 2009c; Davies *et al*. 2013). Interestingly, deficient firing of VTA DAergic neurons is also seen after downregulation of *GDNF* in the Acb (Wang *et al*. 2010a), suggesting that *GDNF* knockdown in the Acb mimics the mesolimbic DA deficiency seen after withdrawal from prolonged excessive alcohol consumption (Barak *et al*. 2011b). Strikingly, this common DAergic deficiency, induced by either viral-mediated *GDNF* downregulation or by withdrawal from excessive alcohol consumption, leads to the same behavioral consequence—a sharp relapse to alcohol seeking and drinking (model; Fig. 7). Thus, rats with downregulated *GDNF* expression in the Acb behave as if they have a long history of excessive alcohol consumption even though they have been exposed to alcohol only a few times, suggesting that endogenous GDNF protects against rapid relapse and escalation to excessive drinking via its effects on DAergic transmission. Furthermore, it is plausible that when DA levels in the Acb decline due to withdrawal from alcohol (Barak *et al*. 2011b), viral-mediated downregulation of *GDNF* in the Acb further exacerbates the mesolimbic DA deficiency, leading to the stronger alcohol seeking and relapse phenotypes that we observed here upon knockdown of the growth factor.

Importantly, while acute alcohol increases DAergic firing in the VTA (Nimitvilai *et al*. 2012), previous studies demonstrated reduced firing of DA VTA neurons after withdrawal from alcohol (Diana *et al*. 1993; Bailey *et al*. 2001; Shen 2003; Shen, Choong & Thompson 2007) using repeated injections of alcohol (Diana *et al*. 1993; Shen 2003; Shen *et al*. 2007) or alcohol liquid diet (Bailey *et al*. 2001), whereas we used voluntary oral consumption of alcohol on a schedule of repeated cycles of drinking and withdrawal, which resembles the drinking pattern of human alcoholics (Koob 2003; Vengeliene *et al*. 2008; Koob & Volkow 2010). Thus, our findings suggest that DA deficiency occurs not only after forced alcohol administration but more importantly under more behaviorally relevant conditions.

Interestingly, Ahmed and Koob suggested that the transition from moderate to excessive drug intake causes an allostatic decrease in the reward system, so that the levels of drug intake must be progressively increased to achieve a rewarding outcome (Ahmed & Koob 1998, 2005). The results presented here, together with previous studies (Wang *et al*. 2010a; Barak *et al*. 2011b), suggest that the deficient VTA DAergic neuron firing and the consequent deficiency of DA release in the Acb are positively correlated with a reduction in reward function after a long history of excessive alcohol consumption. Furthermore, overexpression or local application of GDNF in the mesolimbic system reverses both the DAergic and the alcohol reward deficiencies. Importantly, the effects of alcohol and GDNF on the reward system are distinct, as GDNF on its own is not rewarding (Barak *et al*. 2011b).

Our findings provide evidence that GDNF is an endogenous factor that gates the transition from moderate to excessive alcohol drinking and relapse in the mesolimbic system. Interestingly, GDNF was shown to negatively regulate the reward and intake of other drugs of abuse, including cocaine (Carnicella & Ron 2009; Ghitza *et al*. 2010; but see Lu *et al*. 2009; Airavaara *et al*. 2011). Thus, it is plausible that the protective role of GDNF is not restricted to alcohol and that GDNF can prevent the development of addiction to other drugs. Finally, our results raise the possibility that increasing mesolimbic GDNF levels with medications such as the FDA-approved drug cabergoline, which increases GDNF expression in the VTA (Carnicella *et al*. 2009a) or possibly in the future via gene therapy, can prevent the escalation to excessive alcohol consumption in individuals with a predisposition to develop alcoholism.

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Disclosure

All authors reported no biomedical financial interests or potential conflicts of interest.

Authors Contribution

SB and DR designed the research; SB, JW, SA and SBH performed the research; SB, JW, SA, SBH and DR analyzed the data; APK, JF and KSB provided the AAV-GDNF and consulted on data analysis; and SB and DR wrote the

article. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Downregulation of *GDNF* expression in the NAc does not affect presses on lever that was not associated with reward during operant alcohol self-administration. Rats were trained to press on an active lever for an alcohol reward (10 percent), whereas presses on the other (inactive) lever had no consequences. After achieving a stable baseline operant responding, Adv-shGDNF (shGDNF) or Adv-shSCR (shSCR; 1.3×10^9 TU/ml; total of 3 µl/side) was infused into the NAc of rats to locally downregulate *GDNF* expression (Wang *et al.* 2010a). Line graph depicts the mean ± SEM number of presses on the inactive lever ($n = 7–8$).

Figure S2 Downregulation of *GDNF* expression in the NAc does not affect presses on lever that was not associated with reward during reinstatement and reacquisition of operant alcohol self-administration. Rats were trained to press on an active lever for an alcohol reward (10 percent), whereas presses on the other (inactive) lever had no consequences. After achieving a stable baseline of alcohol (10 percent) self-administration rats underwent 10 days of extinction, after which Adv-shGDNF (shGDNF) or Adv-shSCR (shSCR; 1.3×10^9 TU/ml; total of 3 µl/side) was infused into the NAc of rats to locally downregulate *GDNF* expression (Wang *et al.* 2010a). On days 8 and 9, relapse was assessed: on day 8 post-viral infection, rats had a reinstatement test (alcohol was not delivered following lever presses; measuring relapse to alcohol seeking). The next day rats had a reacquisition test (alcohol was delivered following lever presses; measuring relapse to alcohol consumption). Bar graph depicts the mean ± SEM number of presses on the inactive lever ($n = 7–9$).

Figure S3 Adeno-associated virus 2 (AAV2) containing GDNF infects neurons in the NAc and increases GDNF

mRNA expression. 3 μl/side AAV2-GDNF or control AAV2-GFP (1×10^{12} TU/ml) was infused into the NAc of rats. (A) GDNF levels in the NAc were determined by semi-quantitative RT-PCR on day 21 post-virus infusion. Data are expressed as means ± SEMs of *GDNF*/*GAPDH* expression (*$P < 0.05$; $n = 5$). (B) Immunohistochemistry was performed 21 days post-viral infection with anti-neuronal nuclei (NeuN; a marker of dopaminergic neurons, red) and anti-GFP (for detection of viral infection, green) antibodies. (Scale bar: top, 100 μm; bottom, 20 μm).

Figure S4 Adeno-associated virus 2 (AAV2) containing GDNF infects dopaminergic neurons in the VTA and increases GDNF mRNA expression in the VTA but not in the substantia nigra (SN). 1.2 μl/side AAV2-GDNF or control AAV2-GFP (1×10^{12} TU/ml) was infused into the VTA of rats. (A) GDNF levels in the VTA and SN were determined by semi-quantitative RT-PCR on day 21 post-virus infusion. Data are expressed as means ± SEMs of *GDNF*/*GAPDH* expression. (**$P < 0.005$; $n = 6$). (B) Immunohistochemistry was performed 21 days post-viral infection with anti-tyrosine hydroxylase (TH; a marker of dopaminergic neurons, red) and anti-GFP (for detection of viral infection, green) antibodies. (Scale bar: top, 200 μm; bottom, 50 μm).

Figure S5 *GDNF* mRNA expression levels in the VTA are negatively correlated with alcohol intake. Levels of alcohol intake were measured in the 21st session of intermittent access to 20 percent alcohol two-bottle choice. *GDNF* mRNA expression levels were determined by semi-quantitative RT-PCR, and normalized to *GAPDH*. $n = 14$

Appendix S1. Materials and methods.